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# Regulation of Tissue Growth in Crustacean Larvae by Feeding Regime

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**Abstract.** Growth of the posterior dorsal carapace, the underlying epidermal cells, and the lateral thoraco-abdominal muscle was examined in the second instar *Palaemonetes pugio* under different feeding regimes. Control larvae (continuous feeding) and larvae fed for the first two days of the molt cycle demonstrated a mean molt increment (MI) of 10.6 and 11.1%, respectively. The muscle in these control larvae grew in width by 6.7%. Starved second instar larvae showed a MI of 3.2% and an increase in muscle width of 1.3%. Larvae fed on only one day of the molt cycle had MIs of 5.5–6.6%—values significantly different from that of the control larvae. Muscle growth in partially fed larvae was intermediate (3.9–4.5%) between those of fed and starved larvae. The increase in the density of the epidermal cells was proportional to the MI for the control and starved larvae, and for larvae fed on day 2; larvae fed only on day 1 or day 3 grew less or more, respectively, than the MI predicted from the increase in cell density. The results show that nutritional state is a strong regulator of tissue growth in shrimp larvae.

## Introduction

Food and nutritional state have long been known to affect growth and development of crustacean larvae (Hartnoll, 1982; McConaugha, 1985, for review). Food intake regulates the rate of molting or molt cycle duration (MCD), molt increment (MI, growth at ecdysis), and rate of development in larvae (Knowlton, 1974; McConaugha, 1982; West and Costlow, 1988). In some species, growth, molting, and development are affected differentially by restricted feeding conditions. Several studies have suggested that a hierarchical partitioning of the nutrients for growth, molting, and development may exist, although the mechanism controlling this selection process is not understood (Knowlton, 1974; Anger and Dawirs, 1981; LeRoux, 1982; McConaugha, 1982, 1985; Anger, 1984; West and Costlow, 1988).

Thus far, little is known about the regulatory mechanisms that determine how the level of food consumption may control growth of the integument and tissues. Aspects of growth of the epidermis have been examined in adult shrimp (Tchernigovtzeff, 1965), juvenile crabs (Freeman *et al.*, 1983), larval brine shrimp (Freeman, 1986), and *Daphnia* (Halcrow, 1978). In those studies, the nutritional state and feeding history of the animal were not considered.

To study growth regulation in crustaceans, it will be necessary to determine how the epidermis and muscle—the two tissues with the greatest mass and interaction with the integument—grow during the molt cycle. In this study, instar II *Palaemonetes pugio* larvae were reared under different feeding regimes to examine growth of the epidermis and muscle with respect to feeding and to further define the relationship between the growth of the tissue and the carapace.

## Materials and Methods

Larvae were hatched from egg-bearing females collected locally and maintained individually in the laboratory. The artificial seawater, (Instant Ocean, Aquarium Systems, Ohio), was maintained at 15 ppt and at 24°C (room temperature). Under these conditions, the duration of the larval molt cycle was approximately three days.

Instar I (SI) larvae were fed brine shrimp nauplii, and the water was changed daily. Upon ecdysis to instar II (SII), the larvae were placed in containers with or without food for the appropriate test period (see Results); the water was changed daily. The data were excluded where cannibalism (indicated by partially eaten larvae) occurred.

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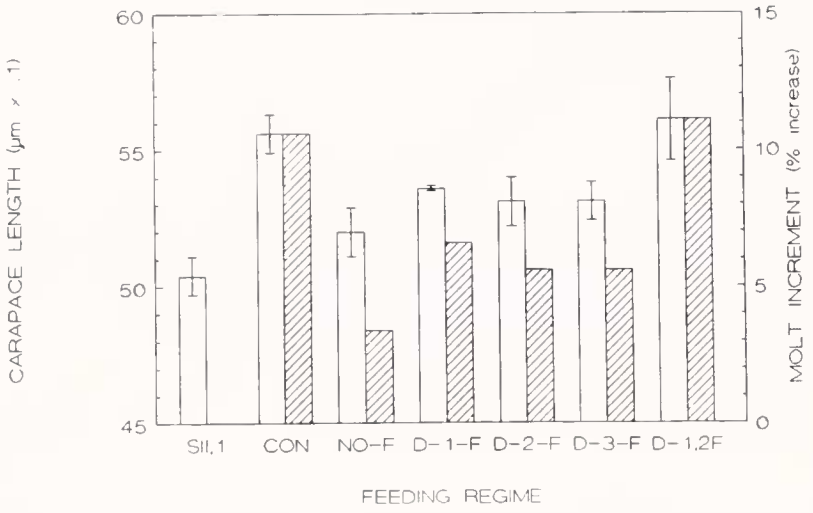


**Figure 1.** Effect of the instar II feeding regime on the carapace length (open bars; left ordinate) and molt increment (diagonal striped bars; right ordinate) of the resulting instar III larvae. The carapace length of day 1, instar II larvae is shown for comparison (SII, 1; single bar). Each bar represents the mean and one standard deviation of 56–162 larvae. Abbreviations for this and all other figures: CON, control larvae fed throughout the molt cycle; NO-F, starved larvae; D-1-F, D-2-F, D-3-F, D-1,2F, larvae fed, respectively, on day 1, 2, 3, or on days 1 and 2.

Measurement of the carapace length (CL) and muscle width (MW) in living larvae was done with a calibrated ocular micrometer. Larvae were immobilized on a slide in a drop of water. The CL was determined by measuring the distance between the posterior edge of the dorsal carapace and a point even with the posterior edge of the orbit. The width of the lateral thoraco-abdominal extensor muscle was measured at the junction of the muscle with the dorsal carapace. The CL and MW determinations were made between 4 and 8 h after ecdysis to instar II ($CL_{SII}$) or instar III ($CL_{SIII}$). These time points are referred to in the Results as day 1, SII or day 1, SIII, respectively. The molt increment (MI) was determined as: $[(CL_{SIII}/CL_{SII}) - 1] \times 100$. Analysis of variance (F-test) was used to determine statistical significance ($P < .05$).

The density of the epidermal cells was measured from specimens fixed in Carnoy's fluid, rehydrated, and stained with the nuclear fluorochrome bisbenzimide (Hoechst 33258, Sigma Chemical Co., St. Louis, Missouri) in phosphate buffered saline (PBS; pH 7.4). The larvae were mounted in PBS or 80% glycerol, with the dorsal carapace up and the long axis of the thorax oriented perpendicular to the axis of the slide. All measurements were made from an image in which the rostrum pointed towards the top of the field of view. The fluorescent image of the epidermal region was captured by a Dage-MTI (Michigan City, Michigan) 67M newvicon video camera mounted on a Leitz Dialux photomicroscope with a PCVISIONplus frame grabber controlled by IMAGEACTIONplus software (both from Imaging Technologies, Inc., Woburn, Massachusetts). A digital rectangle (90 × 109 μm) was superimposed on the posterior dorsal carapace, and the number of nuclei within the rectangle was determined. The cell density was determined on the first and second (and, in some cases, on the third) days of SII, and on the first day of SIII. Because cellular growth leads to expansion in both width and length at ecdysis, the increase in cell number is similar to the potential growth in area of that region of the carapace and therefore approximates the square of the MI in carapace length (Freeman, unpubl.). This "growth potential," or predicted MI (% increase), is defined as: $[(D_{day\ 2}/D_{day\ 1})^{1/2} - 1] \times 100$, where D is the density of epidermal cells in SII on the days indicated.

## Results

The feeding regime markedly affected the MI (% increase in carapace length) of the instar II larvae (Fig. 1). Larvae fed throughout the molt cycle (control) or on the first two days of the molt cycle (D-1, 2F) demonstrated MIs of 10.6 and 11.1%, respectively. These values were significantly greater than those of all other groups. Starved larvae (NO-F) showed a MI of 3.4%, which was significantly lower than those of all other groups. An intermediate level of growth (5–7%) was observed in larvae fed only on day 1, 2, or 3 of the molt cycle. There was no significant change in the MCD of starved or partially fed larvae. Many of the larvae that were starved or fed only on day 3 lived for 5 or 6 days without molting.

Reduced carapace growth of larvae maintained on a restricted feeding regime was presumably a result of less

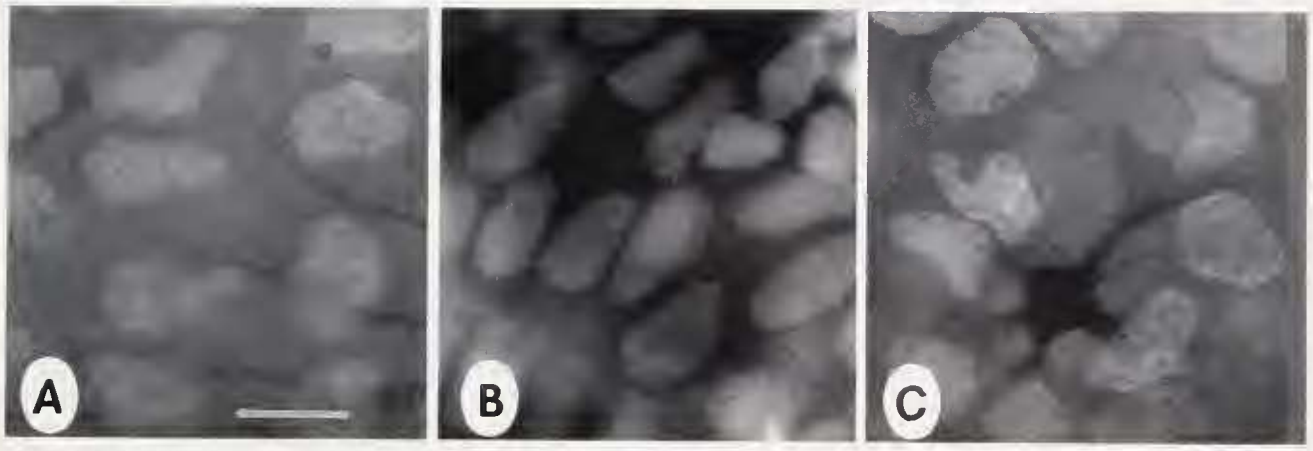


**Figure 2.** The change in density of the epidermal cells in the posterior dorsal carapace during the second and third instars. The nuclei are stained with bisbenzimide and photographed with epifluorescence optics. A. Carapace epidermal cells in a larva at 6 h after ecdysis to instar II (day 1, SII). The density increased during the first day of the instar, reaching the greatest level by day 2, SII (B). The density decreased when the integument expanded after ecdysis to instar III (C). Bar = 25 $\mu$m.

growth of the epidermis which secretes it. To determine if starvation or a restricted feeding regime led to reduced growth of the epidermis, the increase in density of the epidermal cells during SII was determined.

The cell density (nuclei per 90 × 109 $\mu$m area) in freshly molted SII larvae was 18.3 cells (Figs. 2, 3). In controls and larvae fed on days 1 and 2, the cell density rose to 22–23 cells (Figs. 2, 3) for a predicted MI of 11–12%. The densities were greater than those for all groups except those fed on day 1. The density returned to 18 by day 1, SIII. Starved larvae showed the least amount of cell growth (1–2 cells), and the predicted MI (2.7%) was very close to the measured MI (3.4%, Fig. 1). The cell density of starved larvae was significantly different from fed larvae (Control) and larvae fed on day 1, or on days 1 and 2, but not significantly different from those fed on day 2 or day 3.

The predicted MI was similar to the mean measured MI (less than one cell difference) for all groups except larvae fed only on day 1 or day 3 ($r = 0.91$, $P = 0.01$, for all groups). The cell density of larvae fed on day 1 was not significantly different from fed larvae, but was significantly greater than larvae fed on day 2 or 3, or starved. Larvae fed on day 1 demonstrated a MI (6.6%) that was well below the predicted MI (9.4%). Conversely, larvae fed on day 3 grew by 5.6%, which was much greater than the predicted value of 3.0%. Not indicated by the value for larvae fed on day 2 (Fig. 3) was the increase in cell density in larvae feeding on day 2. The cell density value on day 2 (before feeding) was 19.9, which would give a predicted MI of 4.3%. There was an increase of one cell during the day of feeding. Thus, without feeding, these larvae would have shown a growth potential similar to larvae fed on day 3.

Since the integument and muscle presumably grow in a coordinated manner, reduced muscle growth would be expected in larvae reared under restricted feeding conditions. Muscle width was measured on day 1 of instar II and compared to the width on day 1 of instar III. Muscle growth was greatest in the control larvae and larvae fed on days 1 and 2 (Fig. 4). The growth in these two groups was significantly greater than all other groups. Significantly less growth was observed in starved larvae than all other groups. Larvae fed only on days 1, 2, or 3 demonstrated intermediate growth levels that were significantly different from the fed and starved groups, although there was no difference among these groups. The growth in muscle width was highly correlated with the MI predicted from epidermal growth ($r = 0.84$, $P = 0.03$) and the measured MI ($R = 0.92$, $P = 0.01$).

## Discussion

This study clearly shows that growth of the integument and epidermal and muscle tissue is modulated by feeding regime or nutritional state. In addition, larvae in restricted feeding regimes may demonstrate slightly longer molt cycles, an indication that the low food level affected the molt cycle. The data agree with the findings on this and other species of decapod crustaceans (Knowlton, 1974; Hartnoll, 1982; McConaugha, 1985) and demonstrate, furthermore, that growth can be measured at the cellular level.

The high correlation between epidermal growth in the dorsal carapace and carapace length is consistent with previous findings demonstrating that the size of the cuticle after ecdysis is a result of the amount of cell growth in

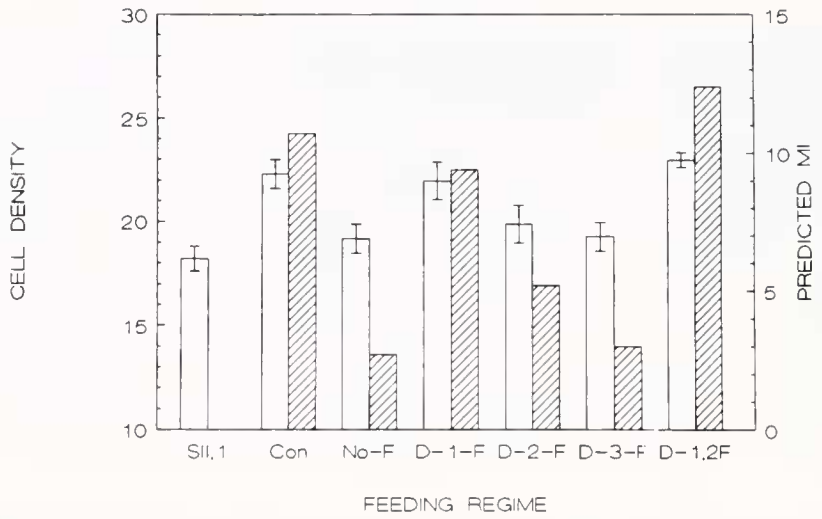


**Figure 3.** Effect of the feeding regime during instar II on the growth of the epidermis in instar II larvae. The cell density at early day 1 was 18 cells (SII, 1, single bar). The cell density of larvae reared in different feeding regimes was measured on day 2 (open bar; left ordinate). For each group the molt increment predicted by the increase in cell density ($[D_{day\ 2}/18]^{1/2} - 1 \times 100$, where $D_{day\ 2}$ is the density on day 2) is also shown (diagonal striped bar, right ordinate). Abbreviations as in Figure 1. Each open bar represents the mean and 1 SD of 10–45 larvae.

the epidermis during the previous molt cycle (Freeman, 1988, unpubl.). The cell density can be used to predict the MI. The results show a close correlation between the tissue growth and cuticular growth for all groups except those fed on days 1 or 3.

The dissimilar MIs measured in larvae fed only on day 1 or 3 cannot be explained by the experiments from this study. Possibly the cell density of larvae fed only on day 1 (measured on day 2) was later reduced by metabolic requirements, such that, at ecdysis, only a 6.6% increase could be realized. Feeding in shrimp larvae on day 1 may be sufficient to reach the point of reserve saturation (Anger and Dawirs, 1981; Anger, 1984), or a threshold for growth and development (West and Costlow, 1988), but it may not be enough to support the optimal amount of growth.

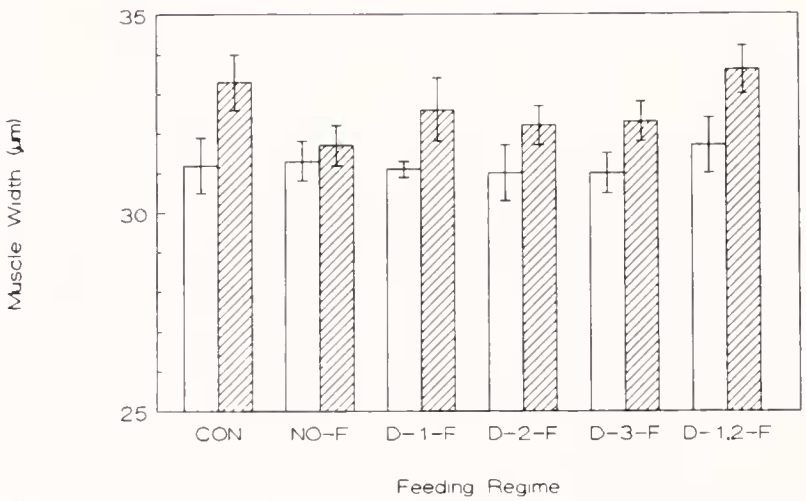


**Figure 4.** Effect of feeding regime during instar II on growth of the lateral thoraco-abdominal muscle. The width of the muscle in larvae in each feeding regime at the beginning of instar II (open bars) is compared to the width of the muscle in that group on the first day of instar III (diagonal striped bars). The difference in the heights of the paired bars represents the amount of growth of the muscle for that group. Abbreviations as in Figure 1. Each bar represents the mean and 1 SD of 56–162 larvae.

The opposite result was observed in larvae fed on day 3; *i.e.*, the actual MI was greater than that predicted. This result may be explained by the reduced food available for growth and metabolism, as predicted by a day 2 cell density equivalent to a predicted MI of 3.0%. If the integument was in a weakened state, as described for epidermal and muscle tissues of starved crab larvae by Anger (1984), then the stretch at ecdysis due to hydrostatic pressure may have overwhelmed the resistance of the new cuticle, along with the epidermis and muscle, resulting in a MI greater than that set by the growth potential. A similar enhanced growth, or stretch, is seen in eyestalkless larvae (Okazaki *et al.*, 1989). Subsequent feeding on day 3 may have provided only enough energy reserves to complete the molt. The day 3 feeding regime spans the critical "point of no return," as suggested by Anger and Dawirs (1981). The larvae that molted may have received food before this point, while those that remained in SII for extended periods before dying may have resumed feeding beyond this point. Tissue degradation and nutrient depletion may not have been reversed by feeding at this time. The results from larvae fed on day 2 suggest that recovery from the starved condition is possible if feeding resumes during the middle of the molt cycle. This period may be the limit beyond which starvation results in tissue degradation and loss of protein (Anger, 1984; McConaugha, 1985).

Preliminary findings suggest that epidermal growth consists of both cell replication and enlargement of divided cells. The contribution of each phase to the growth process is not understood. Analysis of the cell cycle changes in the epidermal cells is necessary to find the control points of the growth process. There may be several control points where nutritional status may be translated into tissue growth. One may be the entrance to mitosis, and another may be the G1-S transition, both of which have been shown to be control points in many cell types (Murray and Kirschner, 1989; Pardee, 1989). Moreover, the growth process may involve entrance of non-cycling cells into the cycling population.

In this study, the epidermis and the muscle were observed to grow in a coordinated manner, in agreement with earlier studies on muscle growth in crustaceans (Bittner and Traut, 1978; Houlihan and El Haj, 1985). Moreover, muscle growth was affected by nutritional stress in a manner similar to that of the epidermis. These findings would argue that a common mechanism controls the coordinated growth of both tissues. Conversely, the epidermis may control growth of the muscle, possibly through cell-cell interactions. These mechanisms are currently being examined.

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